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Identification of flurochloridone metabolites in rat urine using liquid chromatography/high resolution mass spectrometry

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ABSTRACT

It is of great interest to develop strategic methods to enable chemicals' metabolites to be accurately and rapidly screened and identified. To screen and identify a category of metabolites with distinct isotopic distribution, this study proposed a generic strategy using *in silico* metabolite prediction plus accurate-mass-based isotopic pattern recognition (AMBIPR) and library identification on the data acquired *via* the data dependent MS/MS scan of LC-Q Exactive Orbitrap mass spectrometry. The proposed method was evaluated by the analysis of flurochloridone (FLC) metabolites in rat urine sample collected from toxicity tests. Different from the traditional isotopic pattern recognition (IPR) approach, AMBIPR here was performed based on the potential metabolites predicted *via in silico* metabolite prediction tools. Thus, the AMBIPR treated FLC data was only associated with FLC metabolites, consequently not only avoiding great efforts made to remove FLC-unrelated information and reveal FLC metabolites, but also increasing the percent of positive hits. Among the FLC metabolite peaks screened using AMBIPR, 87% of them (corresponding 97 metabolites and 49 biotransformation) were successfully identified *via* multiple MS identification techniques packaged in an established FLC's metabolites library based on Mass Frontier. Noteworthy, 34 metabolites (89%) were identified without distinct naturally isotopic distribution. The universal strategic approach based on background subtraction (BS) and mass defect filtering (MDF) was used to evaluate the AMBIPR and no more false positive and negative metabolites were detected. Furthermore, our results revealed that AMBIPR is very effective, inherently sensitive and accurate, and is easily automated for the rapidly screening and profiling chemicals related metabolites.

1. Introduction

Identification of metabolites of specific chemicals in complex biological specimen has been a challenge, as the metabolites of interest are frequently masked by the high background noises from endogenous or exogenous compounds [1–6]. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) has extraordinary specificity and selectivity for accurate mass identification and has exerted a profound influence on characterizing metabolites from complex biological matrices. LC-HRMS has its capacity in distinguishing chemical-specific metabolites from most isobaric endogenous compounds and in metabolite structure elucidation by determining the elemental composition of precursors and fragment ions [2–4,6–8].

Among high resolution mass spectrometers, LTQ-Orbitrap mass spectrometry seems not to be better in metabolite identification than time-of-flight mass spectrometry (TOFMS), due to its suffering lower detection sensitivity, false negatives, and the resulting slow data acquisition rate [9]. Q Exactive MS, another type of Orbitrap MS, permits a mass scan rate of up to 12 Hz at 17,500 (FWHM) resolution, thus makes up LTQ-Orbitrap's drawback in data acquisition speed [10]. Q Exactive MS enables data-dependent acquisition of MS/MS spectra to be valuable for structural elucidation from a single run.

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It is much challenging to extract potential metabolite-related signals in total ion chromatography from huge mass spectral information acquired through HRMS. The extraction was much time consuming and likely missing those minor metabolites or the metabolites with low MS responses [4,5,7,11,12]. To improve and speed up this process of metabolite detection, some fast, reliable and software-supported MS data-handling approaches are needed. These approaches mainly include isotopic pattern recognition (IPR), background subtraction (BS), mass defect filtering (MDF) and constant neutral loss filtering (CNLF) and characteristic fragment ion recognition (CFIR) [1–4,7,11–14]. Owing to difference of various metabolites in their chemical structure, approaches are usually different in metabolite screening and identification. Therefore, it is more important to determine whether single approach or multiple approaches to be used for specific metabolite, so that many metabolites could possibly be accurately identified as possible. BS + MDF could filter out the vast majority of background signal ions as a strategic/universal technique for metabolite's screening, while IPR, CNLF and CFIR were used as the assistant tool for metabolite characterization and identification [1,3,4,14,15]. BS + MDF possessed inherent characteristics of narrowing down a search range and facilitating metabolite identification, and covered all kinds of analytes, but might lack specificity and generate high false-positives in metabolite screening [3,11,12]. In the subsequent metabolite identification, it usually requires laborious pre-adjustment of the instrument and several LC/MS runs to cover all desired neutral loss and product ion acquisition types.

During the recent ten years, the metabolite identification has been mainly focused on pharmaceuticals and drugs [1,8] and the studies on environmental chemicals were limited. Metabolite profiling and identification are critical for studying deposition and potential toxic effects of environmental chemicals on experimental animals and exploring the potential specific biomarkers for human biomonitoring [15–18]. Compared with pharmaceuticals and drugs, quite a number of environmental chemicals with chlorine, bromine and/or sulfur atoms in their chemical structure, endow them with a distinct isotopic distribution. Therefore, it is possible to uniquely screen and identify these chemicals using IPR. An IPR based on an accurate-mass-based spectral- averaging isotope-pattern-filtering (AMSA-IPF) algorithm was previously reported to extract ion signals containing simple isotope patterns such as those of chlorine- and bromine- containing compounds [19]. This IPR presented much better performance in removing the drug-unrelated ions presented as compared with the universal strategies based on BS and MDF [19], but there still existed the drug-unrelated ions which potentially hamper the subsequent rapid identification of metabolites, especially for the low-response metabolites whose signals, just like in BS and MDF may potentially be masked by the drug-unrelated signals. However, this problem would be avoided if the *in silico* tools were introduced to predict the potential metabolites as templates for IPR. It is worth noting that a large number of experiments showed the good accordance between the experimental and *in silico* data [1,12,20,21], which makes it come true that *in silico* metabolite prediction plus IPR as a generic strategy will be employed for the rapid drug-related metabolites' screening and identification with few false positives and negatives. However, this technique was mainly applied in the fabricated or custom synthesized stable isotopic compounds for the study of drug metabolism [2,22], as well as in the structure elucidation for the naturally isotopic metabolites [1,3,23].

This study aimed to establish and evaluate a strategy based on *in silico* metabolite prediction plus accurate-mass-based isotopic pattern recognition (AMBIPR) and library identification packaging multiple MS identification techniques for screening and identifying the metabolites with a specific isotopic pattern. A pilot study was performed using rat urinary metabolites of flurochloridone (a widely used herbicide) as a representative chemical as well as a performance evaluation of AMBIPR by comparing with the universal method – BS plus MDF.

2. Materials

FLC (purity > 95.5%) was purchased from Jiangxi Anlida Chemical Co., Ltd. (Jiangxi, China). Methanol and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (purity ≥99.0%, LC–MS Ultra grade) and formic acid (purity ≥98.0%, LC–MS Ultra grade) were purchased from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO, USA). Ultra-pure water were purchased from Wastons (A.S. Watson Group Ltd., Hong Kong). Oasis HLB solid-phase extraction (SPE) cartridges (3 ml) were purchased from Waters (Milford, MA, USA).

2.1. Experimental animals and sample collection

Biological experiment was performed based on our previous study [24]. Wistar rats (200–260 g) used in this study were supplied by the Shanghai Institute of Pharmaceutical Industry and all animal studies were performed under the approval of the Institutional Authority for Laboratory Animal Care. Rats were fed with a standard diet for 10 days to adapt to the environment before drug administration. Water was freely available for rats during experiments. FLC was suspended in 0.5% (w/v) sodium carboxymethyl cellulose (CMC-Na) as a vehicle at a concentration of 6.25 mg/ml. Urine was collected at 0 h and 6 h after oral administration of FLC (125 mg/Kg of dose) as control (6–8 ml for each rat) and treatment (2–3 ml for each rat) samples, respectively. After collection, all samples were pooled according to the control and treatment sample types and immediately frozen at −20 °C until chemical analysis.

2.2. Sample treatment

The sample treatment was performed in three steps, which included protein and solid residue precipitation, low-temperature liquid separation followed by SPE. Urine samples (0.5 ml) were added with 1.5 ml acetonitrile and placed into a 4 °C fridge for 20 min after vortex mixing. The low temperature sample were then centrifuged at 15,000 rpm for 5 min. The supernatants were concentrated to dryness and reconstituted with 3-ml mixture of methanol-water (1/9, v/v). The solution was loaded to a HLB SPE cartridge, which was conditioned with 3 ml acetonitrile and 5 ml water consecutively. Methanol-water (3 ml, 1/6, v/v) were employed to wash the cartridge and acetonitrile-water (3 ml, 2/1, v/v) to elute the target metabolites. The eluents were concentrated to dryness and reconstituted with 100 μl acetonitrile and stored at 4 °C for 1 h. All prepared samples were centrifuged for 5 min at 15,000 rpm, the supernatants were used for instrumental analysis.

2.3. Chromatographic separation and MS detection

The analysis was performed using an UltiMate 3000 Hyperbaric LC system coupled to a Q Exactive MS. Chromatographic separation was performed using a Thermo Hypersil BDS C18 column (150 mm × 2.1 mm, 1.9 μm). Mobile phase A (methanol-water, 2/98, v/v, 0.1% formic acid) and mobile phase B (water-methanol, 2/98, v/v, 0.1% formic acid) were utilized at a flow rate of 0.4 ml/min. Mobile phase gradient was as follows: 0–2 min, 95% A; 2–40 min, linear from 95% to 5% A, 40–45 min, and 95% A for equilibration. The temperatures of column oven and autosampler were set at 30 and 4 °C, respectively. Injection volume was 5 μl.